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ABSTRACT

The study described herein was designed to determine if and how a non-toxic, naturally occurring bioflavonoid, galangin, affects growth of human mammary tumor cells. Our previous studies demonstrated that, in other cell types, galangin is a potent inhibitor of the aryl hydrocarbon receptor (AhR), an environmental carcinogen-responsive transcription factor implicated in mammary tumor initiation and growth control. Our results indicated that breast cancer Hs578T cells expressed high levels of constitutively active AhR. Constitutive and environmental chemical-inducible AhR activity was profoundly suppressed by galangin as was cell growth. However, the failure of a-naphthoflavone or FhAhRR transfection to block growth indicated that galangin-mediated AhR inhibition was either insufficient or unrelated to its ability to significantly block cell growth at therapeutically relevant doses. Galangin inhibited transition of cells from the G0/G1 to the S phases of cell growth, likely through the nearly total elimination of cyclin D3. The results suggest that this non-toxic bioflavonoid may be useful as a chemotherapeutic, particularly in combination with agents which target other components of tumor cell cycle and in situations where estrogen receptor-specific therapeutics are ineffective.

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Introduction

It has long been suggested that ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH), contribute to human breast cancer. The preferential targeting of breast tissue by orally administered PAH in rodent breast cancer models supports this contention. Most of the biologic activity of PAH and related dioxins is mediated by the AhR. AhR activation can induce cytochrome P-450 enzymes, proto-oncogenes (e.g. *c-myc, Ha-ras, c-erb-2*) and transcription factors (e.g. NF-κB). Therefore, it is possible that AhR activation plays an important role in the initiation and progression of carcinogenesis by regulating a cascade of intracellular events involving NF-κB, *c-Myc*, and/or other proto-oncogenes. To investigate the role of the AhR in breast cancer development, we originally proposed three aims: Aim 1 is to map the contact domains between AhR and Rel A, aim 2 is to assess AhR regulation of NF-kB activity and c-myc transcription in mammary tumor cells, aim 3 is to characterize AhR regulation of cell cycle components in mammary tumor lines.

Previously, we demonstrated that the AhR and CYP1B1, an AhR-regulated gene, are dramatically up regulated in rodent and human breast tumors. In pursuit of specific aim #2, we investigated the possible consequences of this apparent constitutive AhR activation on the regulation of *c-myc*. In specific, we tested if constitutively active AhR in a human breast cancer cell line regulates *c-myc*, an important breast cancer oncogene that contains six AhR binding sites (AhREs) in its promoter. In 2004, we reported our results that: 1) there is a significant baseline level of wildtype *c-myc* promoter driven reporter activity in these tumor cells which was not affected by inclusion of TCDD, a strong AhR agonist, 2) the baseline reporter activity was not affected by deletion of the NF-κB site, 3) while mutation of single AhRE sites had no effect on baseline reporter activity, mutation of all six sites resulted in a five fold increase in reporter activity; a similar increase in reporter activity was seen when the wildtype reporter construct was co-transfected with an AhR repressor plasmid, 4) *c-myc*-specific real time PCR indicated that AhR repressor transfection increased background levels of endogenous *c-myc* mRNA. These results suggest that the AhR represses *c-myc* transcription and that AhR up-regulation in tumor cells may represent a failed growth feedback mechanism.

This year we continued to work under aim #2. Our work resulted in a mamuscipt to be published in Oncogene this year: The Aryl Hydrocarbon Receptor Constitutively Represses c-myc Transcription in Human Mammary Tumor Cells. We also worked Task #3, which is to use flavonoid to investgate AhR regulation of cell cycle components, such as cycline D3, E and A, in a mammary tumor line Hs578t. Flavonoids are a diverse class of naturally occurring polyphenolic plant compounds, which are consumed daily by humans. Galangin (3,5,7-trihydroxyflavone) belongs to one class of flavonoids known as flavonols and is an aryl hydrocarbon receptor (AhR) inhibitor in some cell types. Studies have shown that AhR, a member of the Per/ARNT/Sim (PAS) family of transcription f actors, plays an important role in cell cycle and apoptosis regulation, and may have a role in the development of breast cancer. Because galangin is an AhR inhibitor in at least some cell types, it seemed plausible that galangin has the potential to block formation of mutagenic metabolites in tumor cells and to regulate human mammary cancer cell growth. The former possibility is supported by the demonstration that galangin blocks *CYP1A1* induction and DNA-adduct formation in a human mammary tumor cell line. The latter possibility, that galangin can alter human tumor cell growth after the transformation process has begun, was investigated herein.

Body

Materials and methods

Reagents

DMEM, RPMI, calcium- and magnesium-free PBS, L-glutamine, penicillin/streptomycin, trypsin-EDTA, and heat inactivated FCS were supplied by Invitrogen (Carlsbad, CA). Galangin, indole-3-carbinol (I3C), and α-naphthoflavone (α-NF) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and dissolved in DMSO (99.9% high-performance liquid chromatography grade, Sigma Chemical Co., St Louis, MO) at concentrations that were 1000-fold higher than the desired final concentration. Insulin was obtained from Sigma.

Cell culture

The ER$^-$ Hs578T human breast cancer epithelial cell line was purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagles medium (DMEM)(Sigma) supplemented with 10% FCS, 10 μg/ml insulin, 50 u/ml penicillin, 50 u/ml streptomycin, and 2 mM L-glutamine. Cells were maintained at subconfluency at 37° C in humidified air containing 10% CO_2 by splitting cultures 1:4 every 3-4 days.

[^{3}H]-Thymidine incorporation

Log phase Hs578T cells (10^3/well) were plated into 96-well tissue culture plates and allowed to adhere overnight. Cells were incubated with 1 μCi ^{3}H-thymidine/well (NEN Life Science Products; Boston, MA) in triplicate wells and dosed with 0.1% vehicle, galangin, I3C, or α-NF for 18 h. Cells were harvested onto filter mats using a PHD cell harvester. ^{3}H-thymidine retained on the filter was detected using a scintillation counter (Becton/Dickinson, San Jose, CA). Triplicates for each data point in each experiment were averaged.

Transient transfections and reporter assays

The *Fundulus heteroclitus AhRR* expression vector was generously provided by Dr. M. Hahn and Dr. S. Karchner (Woods Hole Oceanographic Institution). We and others have shown that this construct is a potent

inhibitor of both human and murine AhR activity. The *pGudLuc6.1*-firefly luciferase reporter construct (*pGudLuc*) construct was kindly provided by Dr. M. Denison (U.C. Davis). AhR-dependent expression of this reporter is driven by four AhREs derived from the *CYP1A1* promoter.

Hs578T cells (10^5/well) were seeded into 6-well culture plates and grown to 70%-80% confluence. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions to transfect cells. The renilla luciferase vector *phRL-TK* (0.5 μg/well) was co-transfected with the 0.1 μg control vector (*pGL3*) or with *pGudLuc* per well. Where indicated, 0.125-0.5 μg of *pcDNA-FhAhRR* or control *pcDNA* were added with or without the reporter construct to the transfection mixture. For each experiment, the amount of total DNA transfected was equilibrated with parental expression vectors. Cells were incubated for 18 hrs, washed twice with phosphate-buffered saline (pH 7.2), and resuspended in 75 μl RPMI prior to luciferase assays. Luciferase activity was determined with the Dual Glo Luciferase system (Promega, Madison, WI) which allowed sequential reading of the firefly and renilla signals. Briefly, cells were lysed in equal volumes of cell lysis buffer (Promega) and RPMI for 20 min, transferred to a 96-well white wall plate, and analyzed using a Reporter Luminometer (Promega). The renilla signal was read after quenching the firefly output, thus allowing normalization between sample wells.

For experiments in which cell growth was assayed after *FhAhRR* or control *pcDNA* transfection, transfected cells were resuspended in RPMI and plated (10^3 cells/well) into 96-well tissue culture plates in triplicate and allowed to adhere overnight. Cells were incubated with 1 μCi ^{3}H-thymidine/well (NEN Life Science Products) and triplicate wells assayed for ^{3}H-thymidine incorporation as described above. Results in the triplicates were averaged for each data point in each experiment.

Cell cycle and apoptosis analyses by flow cytometry

Hs578T cells (10^5/well) were seeded into 6-well tissue culture plates and allowed to adhere overnight. Growth arrest was achieved by washing the cells 3 times with cold PBS before adding supplemented DMEM containing no FCS. In preliminary experiments it was determined that 48 hrs without FCS was required to arrest 80-90% of the cells in G_0/G_1. Less than 5% of these cells stained with trypan blue indicating a high level of viability. Cells were rescued from growth arrest by adding FCS to culture wells (10% final concentration) with

0.1% vehicle, galangin, I3C, or α-NF. Cells were harvested 24 hrs later and analyzed for cell cycle as described below.

Cell cycle analyses and apoptosis quantification were performed by staining cellular DNA with propidium iodide (PI) as we have previously described (58-60). Cells were trypsinized, pelleted, and washed in cold PBS containing 5% fetal bovine serum and 0.02 M sodium azide. Cells were centrifuged for 5 min at 1,000 rpm at 4° C and resuspended in 0.3 ml hypotonic buffer containing 50 μg/ml PI (Sigma), 1% sodium citrate, and 0.1% Triton X-100 and stored protected from light until analysis. Flow cytometry was performed on a Becton/Dickinson FACScan flow cytometer. Data (5,000 events) were collected on both linear and log scales to assess cell cycle and apoptosis respectively.

Western immunoblotting

Cells were scraped into cold PBS and resuspended in $P_{10}EG$ lysis buffer containing 10 mM sodium phosphate, 0.75 mM EDTA, 10% glycerol, 0.125% Triton X-100 and 1.0% protease inhibitor cocktail (Sigma). Cells were lysed after 50 strokes in a Dounce tissue homogenizer (Bellco Glass, Vineland, NJ) and lysis was confirmed by light microscopy. After 15 min on ice cells were centrifuged for 15 min at 13,000 rpm at 4° C. Supernatants were removed and stored at -80° C until Western analysis. Proteins (30 μg/sample) were electrophoresed through 10% SDS-polyacrylamide gels for 1.5h at 100V. Proteins then were transferred to nitrocellulose membranes and membranes blocked for 1 hr at room temperature with 5% non-fat dry milk in PBS with 0.5% Tween-20 (PBS-T). Membranes were probed with the following primary antibodies diluted 1:200 in in PBS-T containing 5% non-fat dry milk: rabbit anti-AhR (Santa Cruz Biotechnology, Santa Cruz, CA), Mouse anti-cyclin A (BD Pharmingen, San Diego, CA), rabbit anti-cyclin D1, rabbit anti-cyclin D3, or rabbit anti-cyclin E (Santa Cruz Biotechnology). Membranes were washed 4 times for 5 min each with PBS-T and incubated for 1 hr at room temperature with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) or goat anti-mouse IgG horseradish peroxidase conjugate (Sigma) at a dilution of 1:20,000 or 1:5,000 respectively prepared in PBS-T containing 5% non-fat dry milk. Membranes were washed extensively with PBS-T and developed with enhanced chemiluminescence. Blots were reprobed up to three

times with a different primary antibody after treating for 15 min with stripping solution (Chemicon, Temecula, CA) and incubating twice for 5 min with blocking solution (Chemicon).

Image analyses

Image analyses were performed on Western immunoblotting autoradiographs that were digitally scanned using a Molecular Diagnostics densitometer. To compare relative band densities between immunoblots, all bands were normalized using β-actin band densities.

Statistical analyses

Statistical analyses were performed with Statview (SAS Institute, Cary, NC). Data from triplicate samples were averaged for each data point for an "n" of 1 in each experiment. Data from a minimum of three experiments are presented as means ± standard errors (SE). One-factor ANOVAs were used to analyze the data. A Fisher PLSD post hoc comparisons test was used to determine significant differences.

Results

Galangin represses constitutive and ligand-induced AhR transcriptional activity

The AhR is expressed at high levels in many rapidly growing human and murine tumors. More specifically, high levels of both cytoplasmic and nuclear AhR characterize rodent and human tumors, including mammary tumors induced with an AhR ligand. These results, and those from several other laboratories, suggest that the AhR is constitutively active in rapidly growing transformed cells. To extend these studies to a human tumor model in which the effects of AhR inhibitors such as galangin can be studied, AhR expression and subcellular localization were determined in a human mammary tumor cell line, Hs578T. As seen *in vivo*, significant levels of AhR protein were detected in both the cytoplasm and the nuclei of Hs578T cells (Figure 1), a result consistent with constitutive AhR activity in this line.

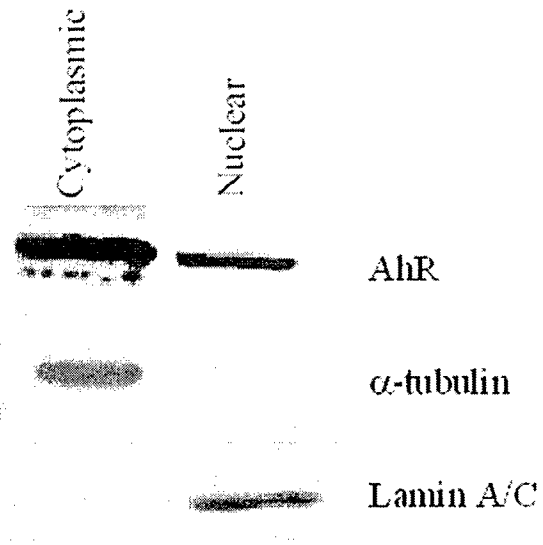


Figure 1: Hs578T cells express nuclear AhR. Cytoplasmic and nuclear cell extracts prepared from subconfluent monolayers of malignant, estrogen receptor negative Hs578T cells were analyzed by western immunoblotting with AhR-specific antibody following SDS-PAGE. Blots were stripped and re-probed for lamin A/C and α-tubulin to confirm purity of the nuclear and cytoplasmic cell fractions respectively. Representative data from a total of 3 experiments are shown.

If this nuclear AhR were indeed constitutively active, it would be predicted that transient transfection of an AhR-driven luciferase reporter construct (*pGudLuc*) would result in significant levels of background transcriptional activity and that this activity would be inducible with AhR ligands and inhibitable with AhR competitive inhibitors, including galangin. To test these predictions, Hs578T cells were transiently transfected with the renilla luciferase plasmid *phRL-TK* to control for transfection efficiency and with control *pGL3* plasmid or *pGudLuc* plasmid and treated with vehicle, one of two AhR inhibitors (galangin; α-naphthoflavone/α-NF), or an AhR agonist (indole-3-carbinol/I3C). Renilla and firefly luciferase activities were assayed 18 hours later.

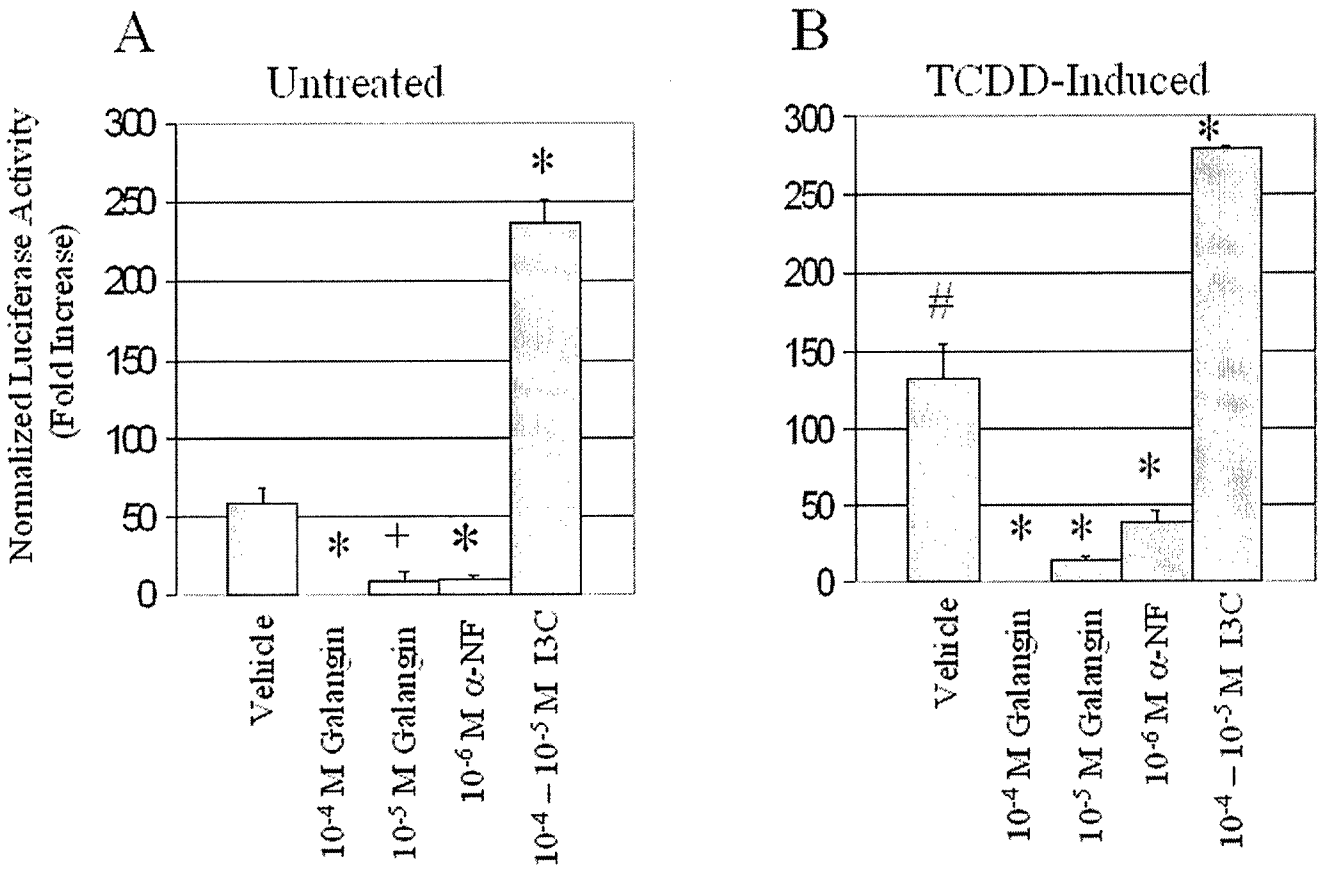


Figure 2: Galangin inhibits AhR-dependent pGudLuc reporter activity. Hs578T cells were left untransfected or were transfected with 0.5 μg/well renilla luciferase vector *phRL-TK* and 0.1 μg control *pGL3* or *pGudLuc* vector per well and treated with 10^{-4}-10^{-5} M galangin, 10^{-4} -10^{-5} M I3C, or 10^{-6} M α-NF in the absence (A) or presence (B) of 10^{-9} M TCDD. Cells were harvested 18 hrs later and luciferase activity assayed. Firefly luciferase activity was normalized to renilla activity in each experiment. (A) Data pooled from 4-16 experiments are presented as the average fold increase relative to non-transfected cells + standard error. An asterisk (*) indicates a significant difference relative to vehicle-treated controls, $p<0.02$. A cross (+) indicates $p=0.056$. (B) Data pooled from 4-16 experiments are presented as the average fold increase relative to non-transfected cells + standard error. An asterisk (*) indicates a significant difference relative to vehicle-treated controls, $p<0.02$. A pound sign (#) indicates a significant increase in activity relative to untreated, *pGudLuc*-transfected controls.

As predicted, transfection with *pGudLuc* increased normalized luciferase activity approximately 50-fold relative to *pGL3*-transfected controls in this series of experiments (Figure 2A). Addition of 10^{-4} M galangin completely blocked the constitutive level of reporter activity ($p<0.02$). At a lower doses (10^{-5} M) galangin tended to decrease the activity, although the data did not reach statistical significance in the three experiments

performed ($p=0.056$). A synthetic flavonoid, α-NF (10^{-6} M), previously shown to block AhR activity (66, 67), similarly reduced constitutive *pGudLuc* activity ($p<0.02$). As expected from previous studies (50), I3C, an AhR agonist, significantly induced *pGudLuc* reporter levels. A similar profile was seen when AhR activity was induced with 10^{-9} M TCDD (Figure 2B). That is, TCDD significantly increased the baseline level of *pGudLuc* activity (Figure 2B, first histogram) relative to untreated controls (Figure 2A, first histogram) while 10^{-4}-10^{-5} M galangin or 10^{-6} M α-NF significantly blocked this induction ($p<0.02$). I3C, together with TCDD, resulted in the greatest increase in *pGudLuc* activity. These data demonstrate that both galangin and α-NF can suppress constitutive and TCDD-induced, AhR-dependent transcriptional activity in a human mammary tumor cell line.

Galangin inhibits Hs578T cell growth

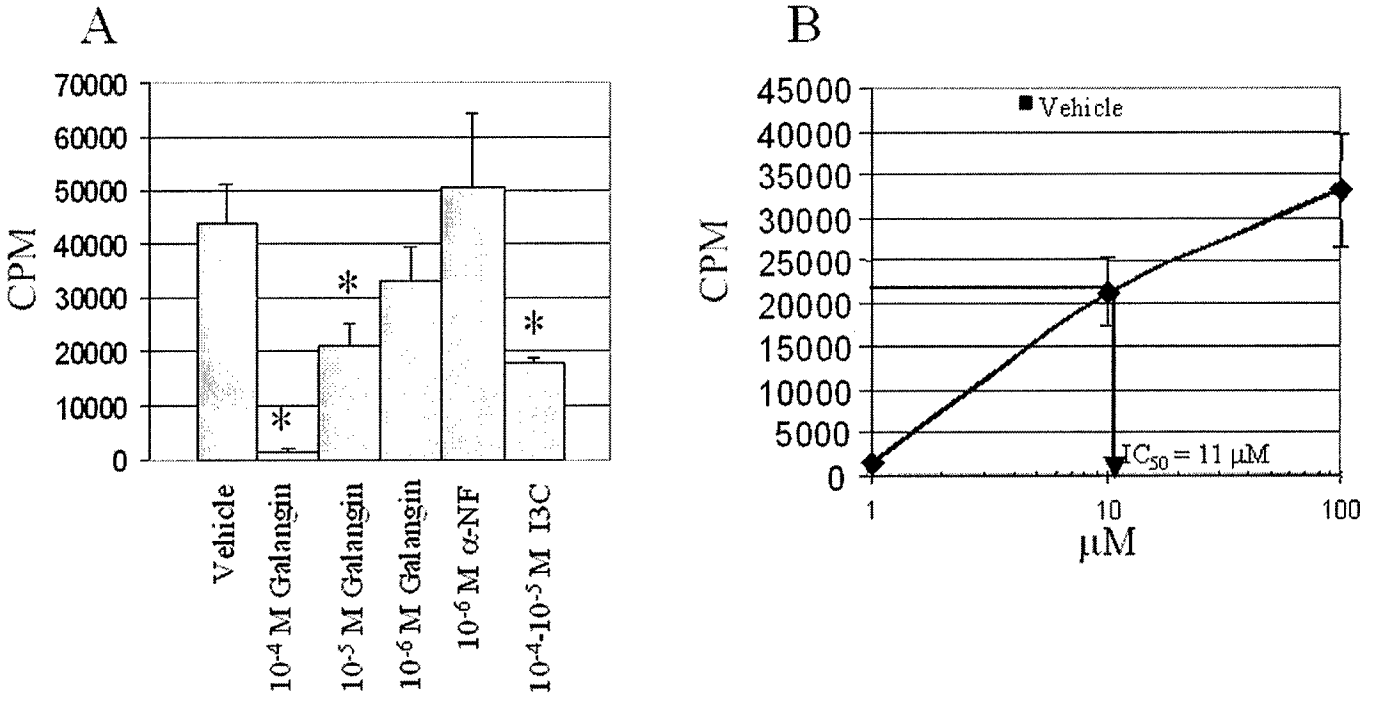


Figure 3: Galangin inhibits proliferation of Hs578T breast cancer cells. Hs578T cells were treated in triplicate with vehicle, 10^{-4}-10^{-6} M galangin, 10^{-4}-10^{-5} M I3C (pooled for statistical purposes), or 10^{-6} M α-NF and grown in ^{3}H-thymidine-containing media for 18 h. Triplicates were averaged for each point in each experiment. (A) Data are pooled from 5-8 experiments and presented as the mean CPM + standard error. An asterisk (*) indicates a significant decrease in ^{3}H-thymidine incorporation relative to vehicle controls, $p<0.04$. (B) Data obtained with galangin as above were replotted to determine the IC_{50}. A square indicates incorporated CPM in vehicle-treated cells (44,181 + 7,381).

Since molecular manipulation of AhR activity can affect cell growth (40, 44), the ability of galangin, α-NF, and I3C to alter Hs578T cell growth was studied. Treatment of Hs578T cells with these three compounds did not affect cell viability as assessed by trypan blue exclusion (>95% trypan blue negative at all doses tested). Addition of 10^{-4}-10^{-5} M galangin significantly ($p<0.04$) reduced cell growth as measured by ^{3}H-thymidine incorporation (Figure 3A). At 10^{-6} M, galangin reduced ^{3}H-thymidine incorporation by 25%, although this reduction was not statistically significant. Overall, the IC_{50} of galangin under these conditions was 11 μM (Figure 3B), a result that compares favorably with concentrations of tamoxifen required to inhibit growth of ER^{+} mammary tumor cells by 50% (i.e. 31 μM). Interestingly, α-NF, which was shown to be a potent AhR inhibitor in this cell line (Figure 2), had no effect on Hs578T cell growth. Consistent with previous studies in ER^{+} cells I3C significantly reduced ^{3}H-thymidine incorporation.

The ability of both an AhR antagonist (galangin) and an AhR agonist (I3C) to suppress cell growth, and the failure of a second AhR antagonist (α-NF) to affect growth, suggested that AhR down-regulation is either not involved or is insufficient for galangin-dependent growth inhibition. Since pharmacologic agents such as galangin may have multiple biologic activities, a second approach, transfection with an AhR-specific repressor, was taken to confirm that AhR down-regulation in and of itself is not sufficient to alter Hs578T cell growth. An evolutionarily conserved AhR repressor (AhRR) specifically blocks AhR-dependent *CYP1A1* activity by competing for the AhR binding partner ARNT and by blocking binding of this complex to recognition sequences in target genes. Notably, AhRR derived from killifish (*F. heteroclitus*) inhibits both human and mouse AhR-dependent transactivation in an AhR-specific manner. In our hands, the *F. heteroclitus AhRR* (*FhAhRR*) expression plasmid is more effective at suppressing *pGudLuc* activity in Hs578T cells than a human *AhRR* expression construct. Therefore, the *FhAhRR* construct was used to determine if inhibition of AhR activity is sufficient to suppress Hs578T cell growth.

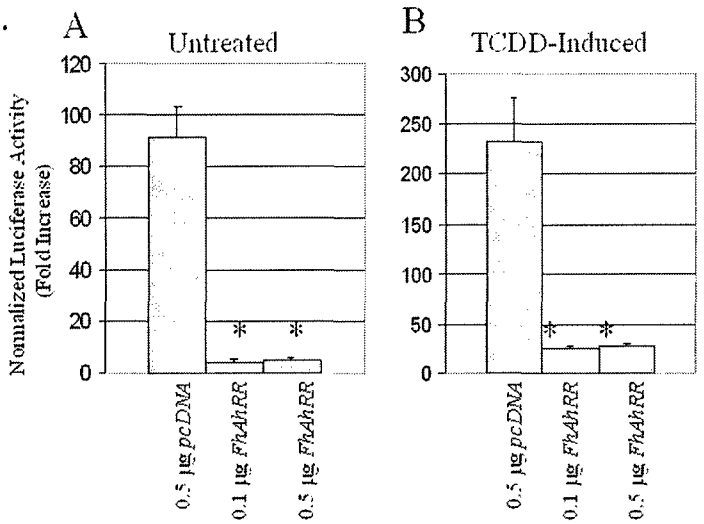


Figure 4: AhR Repressor (*FhAhRR*) inhibits AhR-dependent *pGudLuc* reporter activity. Hs578T cells were left untransfected or were transfected with 0.5 µg/well renilla luciferase vector *phRL-TK* and 0.1 µg *pGudLuc*/well together with 0.5 µg control vector (*pcDNA*), 0.1 µg *FhAhRR*, or 0.5 µg *FhAhRR* in the absence (A) or presence (B) of 10^{-9} M TCDD. Cells were harvested 18 hrs later and luciferase activity assayed. Firefly luciferase activity was normalized to renilla activity in each experiment. Data pooled from 6 experiments are presented as the average fold increase relative to non-transfected cells + standard error. An asterisk (*) indicates a significant difference relative to pcDNA-transfected controls, $p<0.001$.

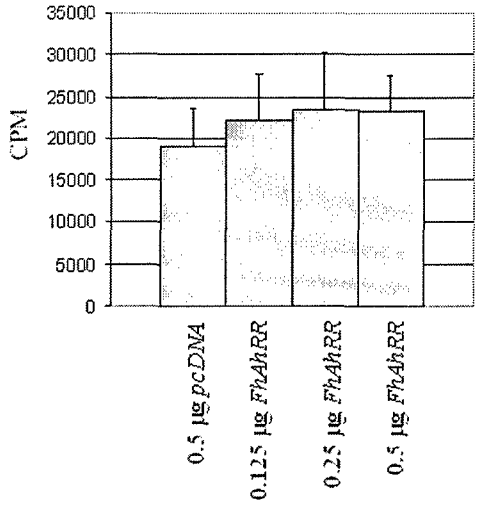
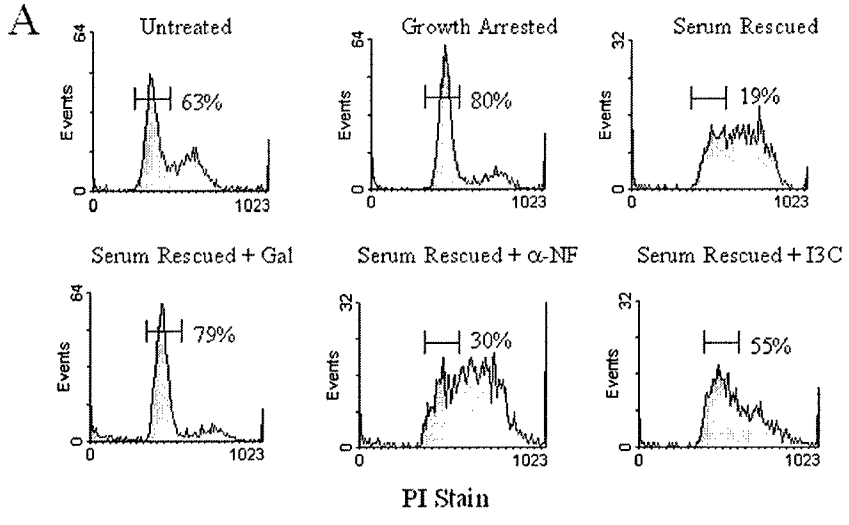


Figure 5: AhRR does not inhibit proliferation of Hs578T breast cancer cells: Hs578T cells were transfected with control *pcDNA* vector or with *FhAhRR* plasmid as in Figure 4, plated in triplicate in 96 well plates, and allowed to adhere overnight before addition of ^{3}H-thymidine. Cells were harvested 18 hrs later and assayed for ^{3}H-thymidine incorporation. Triplicates were averaged in each experiment. Data are pooled from 3 experiments and are presented as the mean CPM + standard error. There were no statistical differences between groups.

Hs578T cells were transiently transfected with *FhAhRR* or control *pcDNA* either with *pGudLuc*, to confirm *FhAhRR* activity, or without *pGudLuc* to evaluate cell growth. Transfection with *FhAhRR* significantly reduced both the constitutive (Figure 4A) and the TCDD-inducible (Figure 4B) *pGudLuc* reporter activity in transfected Hs578T cells confirming the potent inhibitory activity of ectopically expressed AhRR. However, *FhAhRR* transfection had no effect on ^{3}H-thymidine incorporation (Figure 5). These results demonstrate that AhR repression is not sufficient to effect growth inhibition in this cell line. It is concluded that galangin's ability to inhibit cell growth either doesn't involve the AhR or is mediated by AhR suppression together with other activities.

Galangin blocks G_0/G_1 to S transition



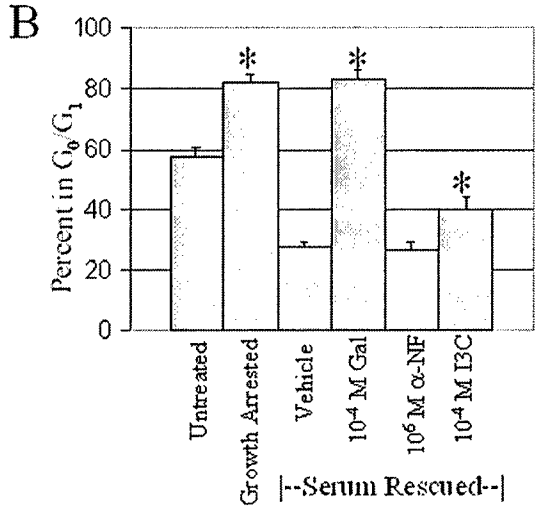


Figure 6: Galangin and I3C block Hs578T cells progression from G_0/G_1 into cell cycle. Hs578T cells were synchronized by serum deprivation for 48 hrs followed by rescue with 10% serum. As indicated, 10^{-4} M galangin (Gal), 10^{-6} M α-NF, or 10^{-4} M I3C were added to triplicate wells at the time of serum rescue. Cells were harvested 24 hrs later and assayed for DNA content by PI staining and flow cytometry. Data from triplicate wells were averaged in each experiment. (A) Flow cytometry histograms from one representative experiment are presented. (B) Data pooled from 3-6 experiments are presented as the mean percentage of cells in G_0/G_1 + standard error. An asterisk (*) indicates a significant increase relative to serum starved, vehicle treated cultures, $p<0.003$.

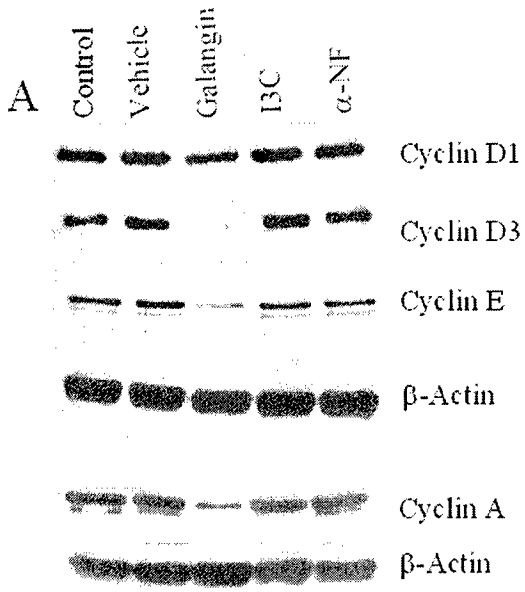
To determine the stage(s) of cell cycle at which galangin blocks growth, Hs578T cells were synchronized by serum deprivation for 48 hours and then serum rescued in the presence of galangin, α-NF, or I3C. DNA content was assayed 24 hours after serum rescue by propidium iodide (PI) staining and flow cytometry. Approximately 60% of the cells growing in log phase were in the G_0/G_1 phase of cell growth at any given time (Figure 6A and 6B). Growth arrest induced by deprivation of serum significantly ($p<0.003$) increased the number of cells in G_0/G_1 to approximately 80%. Addition of serum initiated cell cycle as indicated by a decrease in the number of cells in G_0/G_1 to approximately 20%. However, this decrease in G_0/G_1 cells was not seen when serum was added in the presence of 10^{-4} M galangin. As expected from its failure to affect growth of non-synchronized cells (Figure 3), 10^{-6} M α-NF had no effect on the number of cells exiting G_0/G_1 after serum rescue (Figure 6). I3C (10^{-4} M) partially but significantly ($p<0.003$) inhibited transition of cells from the G_0/G_1 into the S phase of cell cycle after serum rescue.

Growth-arrested, serum-rescued, and flavonoid-treated cells also were assayed for apoptosis as measured by the presence of a sub G_0/G_1 peak as we have described. Regardless of treatment, less than 8% of the cells were apoptotic and no differences were seen between groups. These data indicate that galangin is non-toxic and that it blocks the transition of Hs578T cells from the G_0/G_1 to the S phase of cell growth.

Galangin down-regulates cyclins D3, E, and A

Cell cyclins tightly regulate the transition of cells through the phases of cell cycle. The D cyclins are upregulated at the initiation of cell cycle and drive cells from the G_0/G_1 to the S phase of growth in part through Rb phosphorylation. Cyclin E is upregulated by E2F released from Rb during the late phases of G_1 and, once

complexed with Cdk2, commits the cell to cell division. Cyclin A functions both in the S and M phases of cell cycle. Dysregulation of each of these cyclins has been associated with mammary tumorigenesis. To determine at what level(s) galangin effects growth inhibition, Hs578T cells were left untreated or were treated with 10^{-4} M galangin, 2.5 x 10^{-4} M I3C, or 10^{-5} M α-NF and assayed for cyclin D1, D3, E, and A expression 18 hours thereafter.



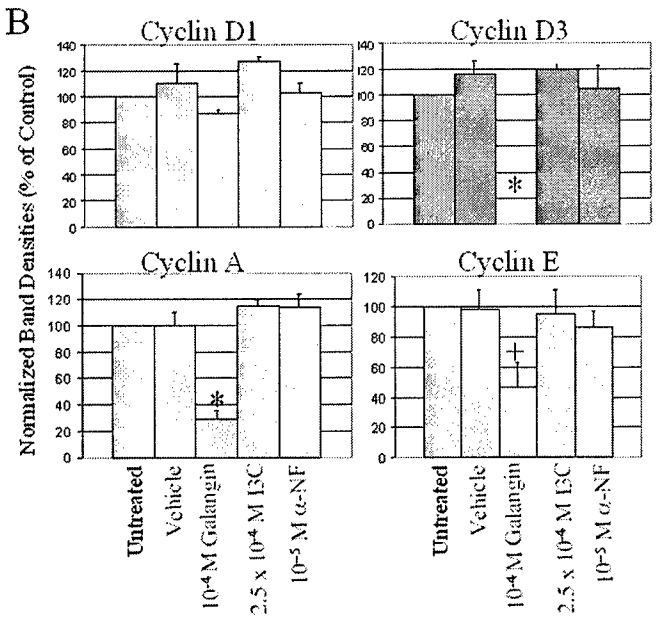


Figure 7: Galangin down-regulates expression of cyclins D3, A, and E. Hs578T cells were left untreated or were treated with vehicle, 10^{-4} M galangin, 2.5 x 10^{-4} M I3C, or 10^{-5} M α-NF and assayed for cyclin D1, D3, E, and A expression 18 hours thereafter by western blotting. Blots were stripped and reprobed with β-actin-specific antibody to control for sample loading. (A) Data from one

representative experiment from a total of 3 experiments are presented. (B) Cyclin band densities were normalized with β-actin band densities and then expressed as a percentage of β-actin normalized cyclin expression in untreated cultures. Data are pooled from 3 experiments and expressed as the percent of control of the respective normalized band densities + standard errors. An asterisk (*) indicates a significant decrease in cyclin expression relative to vehicle controls, $p<0.001$. A cross (+) indicates a significant decrease, $p<0.02$.

Although galangin tended to decrease cyclin D1 expression, the data did not reach statistical significance in this series of experiments (Figure 7A and 7B). However, expression of cyclin D3 was nearly undetectable in galangin-treated cells. Furthermore, galangin significantly reduced expression of cyclins A ($p<0.001$) and E ($p<0.02$). Since cyclins A and E function downstream of cyclin D3, these data are consistent with the cell cycle data (Figure 6) and support the hypothesis that galangin blocks transition of cells from G_0/G_1 into S phase by profoundly down-regulating at least cyclin D3. As in previous experiments, no overt toxicity (i.e. uptake of trypan blue) was noted following galangin treatment.

In contrast, neither I3C nor α-NF significantly affected expression of the cyclins assayed herein (Figure 7A and 7B). The failure of I3C to inhibit expression of these cyclins, while clearly affecting cell growth (Figures 3 and 6), suggests its ability to interfere with other components of the cell cycle machinery not assayed here, e.g. cyclin-CDK activity.

Key Research Accomplishments

1. Western blotting for AhR in cells of an ER$^-$ malignant breast cancer line (Hs578T) demonstrated constitutive AhR activity in this line.

2. To determine if this nuclear AhR was likely to be enforcing constitutive transcription of an AhR target gene, Hs578T cells were transfected with the pGudLuc reporter which contains four AhR binding sites (AhRE) derived from the promoter of CYP1A1, an AhR-regulated gene. Significant levels of background luciferase activity were noted in the transfected cells. This activity increased approximately 3 fold following the addition of the potent AhR ligand, TCDD.

3. Galangin significantly reduced both the constitutive and TCDD-induced luciferase activity without affecting cell viability confirming that galangin is an AhR inhibitor and that background levels of reporter activity are likely due to constitutive AhR activity in this human tumor line.

4. To determine if this AhR inhibitor could alter tumor cell growth, Hs578T cells were treated with 10^{-4} M galangin and evaluated for [3H]-thymidine incorporation. Indeed, galangin significantly reduced cell growth . Analysis of DNA content by flow cytometry indicated that galangin blocked cell cycle at the G1 to S transition. This growth inhibition correlated with a decrease in cyclin D3, E, and A.

5. We find that a naturally occurring, non-toxic bioflavonoid, galangin, effectively suppresses proliferation of an ER$^-$ cell line through down-regulation of cyclins D3, E, and A. While galangin inhibits the activity of the AhR, a transcription factor implicated in the initiation and growth of mammary tumors, AhR inhibition was either not required or not sufficient to suppress growth of this cell line. These results suggest that this bioflavonoid may represent a useful therapeutic for the treatment of ER$^-$ mammary tumors and should complement the effects of therapeutics which target other dysregulated components of the cell cycle machinery.

Reportable Outcomes

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Flavonoid inhibition of mammary tumor growth
-Poster presentation, Era of Hope, Philadelphia, PA. 2005

Abstract of Era of Hope 2005

Flavonoid inhibition of mammary tumor growth

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Flavonoids are a diverse class of naturally occurring polyphenolic plant compounds, which are consumed daily by humans. Galangin (3,5,7-trihydroxyflavone) belongs to one class of flavonoids known as flavonols and is an aryl hydrocarbon receptor (AhR) inhibitor. Studies have shown that AhR, a member of the Per/ARNT/Sim (PAS) family of transcription f actors, plays an important role in cell cycle and apoptosis regulation, and may have a role in the development of breast cancer. Here we investigate if galangin can alter human tumor cell growth through AhR.

Hs578T cells, a human breast cancer epithelial cell line is cultured in manufacture's recommend medium and were harvested for Western blots. Results showed that AhR was constitutively active in Hs578T cells, as was expressed in large amount in nucleus. *pGudLuc* reporter was used for luciferase assay. It has been reported that AhR-dependent expression of *pGudLuc* reporter is driven by four AhREs derived from the *CYP1A1* promoter. Our luciferase assay showed that transfection with *pGudLu* in Hs578T cells increased normalized luciferase activity approximately 50-fold, which confirmed the constitutive activity of AhR. Addition of 10^{-4} M galangin significantly reduced the constitutive level of reporter activity ($p<0.001$). While TCDD significantly increased *pGudLuc* activity relative to untreated controls ($p<0.001$), galangin can significantly blocked this induction ($p<0.02$). [^{3}H] Thymidine incorporation showed that addition of 10^{-4} M galangin significantly reduced cell growth ($p<0.04$). To determine the stage(s) of cell cycle at which galangin functions, Hs578T cells were synchronized by serum deprivation and then serum rescued in the presence of galangin. DNA content was then assayed by propidium iodide staining and flow cytometric analysis. Data indicated that 10^{-4} M galangin completely blocked transition from G_0/G_1 into cell cycle after serum rescue($p<0.0001$).

We conclude that galangin, has the potential to block formation of mutagenic metabolites in and/or to inhibit growth of human cancer cells. Its action may include the inhibition of AhR activity. Our research will continue to focus on galangin as a potential drug to arrest breast cancer cell growth and thus may play an important role in breast cancer therapy.

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